

PCR-RFLP Analysis of *Bactrocera dorsalis* (Tephritidae: Diptera) Complex Species Collected in and Around the Ryukyu Islands of Japan Using the Mitochondrial A-T Rich Control Region

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Abstract: In order to examine the origin of fruit flies of the *Bactrocera dorsalis* complex occasionally trapped on the Ryukyu Islands of Japan, restriction banding patterns of the PCR-amplified mitochondrial A-T rich control region were compared among 513 individuals trapped at 16 localities on the Ryukyu Islands, Taiwan, the Philippines, Vietnam, and Thailand, and detected in fruits imported from the Philippines and China that were intercepted at Narita International Airport. Of a total of 44 haplotypes recognized using the restriction enzymes *Dra* I and *Ssp* I, only three and six haplotypes were detected from the Okinawa and Sakishima regions in the central and southwestern part of the Ryukyu Islands, respectively. Although the former population was composed of haplotypes common among other Asian countries excluding the Philippines, the latter population indicated a close relationship with the Philippines. Thus, there must be two major migration routes into the Ryukyu Islands. This result is consistent with our previous study that *B. philippinensis*, indigenous to the Philippines, may fly into the Sakishima region. In addition, the present study further indicated that one of the two haplotypes corresponding to *B. philippinensis* was widely distributed in Taiwan, mainland China, Vietnam, and Thailand, although the haplotype was not abundant in these areas. This phenomenon suggests the possibility that this species might also have invaded these countries.

Key words: Invasion, fruit flies, *Bactrocera dorsalis*, *B. philippinensis*, mitochondrial DNA haplotype

Introduction

The *Bactrocera dorsalis* complex includes several invasive polyphagous pest species of international significance (CLARKE *et al.*, 2005). Of these, the range of the oriental fruit fly, *Bactrocera dorsalis* (Hendel), extensively expanded in the last century from the Southeast Asia to the Pacific region (WHITE and ELSON-HARRIS, 1992; DREW and HANCOCK, 1994) and even part the North America (ANON., 1987). This species also invaded subtropical islands in the Ryukyu Islands of Japan in the beginning of the 20th century (NAWA, 1919). Although the species was eradicated from all Japanese territories in 1986 (YOSHIZAWA, 1997), precautionary measures are operative in this country to detect and prevent colonization of migrant insects because neighboring countries still remain infested.

In order to determine the origin of fruit flies of the *B. dorsalis* complex occasionally caught in traps in the Ryukyu Islands, we have tried to compare genetic structures among populations collected in and around this area (NAKAHARA *et al.*, 2008; MURAJI *et al.*, 2008). In the analyses using nucleotide sequences and PCR-RFLP of the mitochondrial 12S ribosomal DNA (12S rDNA), we indicated that the mitochondrial DNA (mtDNA) of most of the insects trapped in the Sakishima region of the southwestern part of the Ryukyu Islands is the same as that of *B. philippinensis*, distributed in the Philippines (MURAJI *et al.*, 2008). Based on circumstantial evidences, we concluded that they must have flown directly from the Philippines to the Sakishima region, presumably due to meteorological factors occurring in these regions. However, because the 12S rDNA marker is not so sensitive, we could not specify the origin of many other insects trapped in the Ryukyu Islands.

The A-T rich control region of mtDNA is considered to control replication of the mtDNA molecule. This region is known to be highly polymorphic, much more so than other mtDNA regions. In this study, we examined PCR-RFLP patterns of the A-T rich control region using 513 specimens collected at 16 localities in the Ryukyu Islands, Taiwan, the Philippines, Vietnam, and Thailand, and detected in fruits imported from the Philippines and China that were intercept-

Table 1. Specimens used in this study

Locality	Populations	Date	Number of specimens	Collection
Ryukyu Islands (Okinawa Prefecture, Japan)				
Okinawa region				
Okinawa-jima Island	Okinawa	2000. X. 13–2002. V. 10	42	*1
Kume-jima Island		1998. XI. 4–1999. VIII. 12	2	*2
Sakishima region				
Shimoji-jima Island	Sakishima	2003. VI. 26	1	*1
Ishigaki-jima Island		1999. V. 26–2003. VIII. 21	8	*1
Taketomi-jima Island		1999. VI. 24	1	*1
Iriomote-jima Island		1999. VII. 22	1	*1
Hateruma-jima Island		2003. VIII. 2	4	*1
Mainland China				
Detected from fruits intercepted at Narita International Airport	China	2000. VII. 18–2006. IX. 11	44	*3
Taiwan				
Jhongheng District, Keelung	Keelung	2004. XI. 28	13	Nakahara
Shihlin District, Taipei	Taipei	2002. X	64	Kaneda
Shihlin District, Taipei		2000. XI. 8	21	Iwaizumi
Fengyuan City, Taichung County	Taichung	1999. VI. 16–VII. 4	22	Tokihiro
Taitung City, Taitung County	Taitung	2004. XI. 25–26	48	Nakahara
Gushan District, Kaohsiung	Kaohsiung	2004. XI. 23–24	47	Nakahara
Vietnam				
Ho Chi Minh City	Vietnam	2001. III. 21–28	23	Yoneda
Thailand				
Chiang Mai City	Thailand	2000. II. 8	19	Sakanouchi
Bangkok City		2000. IX. 29–X. 12	20	Iwaizumi
Philippines				
Davao City	Davao	2002. XII. 1–3	51	Miyazaki
Detected from fruits intercepted at Narita International Airport	Philippines	1998. XII. 1–1999. VII. 18	82	*3

Specimens denoted by * were provided by the Okinawa Prefectural Plant Protection Center (*1), the Naha Plant Protection Station (*2), and the Narita International Airport Branch of the Yokohama Plant Protection Station (*3).

ed at Narita International Airport. Based on the results, we discuss the relationships between flies from Ryukyu Islands and neighboring countries.

Materials and Methods

Specimens

The materials used in this study are listed in Table 1. Specimens from China and the Philippines were detected from imported fruits intercepted at Narita International Airport. All others were collected using traps with host plants or the male attractant methyl eugenol. Specimens collected in the Ryukyu Islands were pooled and treated as two populations, Sakishima and Okinawa, according to their geographic origins, because only a small number of samples were available from respective sampling sites in this area. Many of these specimens were also used to analyze mitochondrial 12S rDNA (MURAJI *et al.*, 2008). Specimens were stored in 99.9% ethanol until DNA extraction. Discrimination of the *B. dorsalis* complex from other *Bactrocera* species was based on external morphology (DREW and HANCOCK, 1994).

Experimental procedures

Template DNA for PCR was extracted from individuals using a Genomic Prep Cell & Tissue DNA Isolation Kit (Amersham Biosciences, UK) and dissolved in 200 μ l sterilized water. Amplification of the A-T rich control region of

Table 2. Restriction fragment patterns of the PCR amplified fragment of the mitochondrial A-Trich control region sequences

Restriction enzymes	RFLP pattern	Restriction fragment lengths (kbp)
<i>Dra</i> I	A	0.52, 0.36, 0.32, 0.20, 0.07
	B	0.36, 0.36, 0.32, 0.20, 0.15, 0.07
	C	0.36, 0.36, 0.22, 0.20, 0.15, 0.09, 0.07
	D	0.79, 0.36, 0.20, 0.07
	E	0.52, 0.36, 0.24, 0.20, 0.08
	F	0.52, 0.40, 0.32, 0.20, 0.07
	G	0.52, 0.32, 0.31, 0.20, 0.07
	H	0.40, 0.36, 0.32, 0.20, 0.15, 0.07
	I	0.36, 0.25, 0.20, 0.17, 0.16, 0.15, 0.10, 0.07
	J	0.36, 0.36, 0.32, 0.20, 0.12, 0.07
	K	0.36, 0.36, 0.20, 0.17, 0.16, 0.15, 0.07
	L	0.36, 0.36, 0.22, 0.20, 0.12, 0.09, 0.07
	M	0.47, 0.36, 0.36, 0.20, 0.07
	N	0.47, 0.36, 0.31, 0.20, 0.10, 0.07
<i>Ssp</i> I	A	0.31, 0.25, 0.23, 0.14, 0.13, 0.11, 0.09, 0.06
	B	0.31, 0.25, 0.21, 0.14, 0.13, 0.11, 0.09, 0.06
	C	0.34, 0.30, 0.25, 0.14, 0.13, 0.09, 0.06
	D	0.45, 0.25, 0.23, 0.13, 0.11, 0.09, 0.06
	E	0.45, 0.25, 0.21, 0.13, 0.11, 0.09, 0.06
	F	0.35, 0.25, 0.23, 0.14, 0.13, 0.11, 0.09, 0.06
	G	0.31, 0.28, 0.23, 0.14, 0.13, 0.11, 0.09, 0.06
	H	0.31, 0.25, 0.25, 0.14, 0.13, 0.11, 0.09, 0.06
	I	0.31, 0.23, 0.14, 0.13, 0.13, 0.12, 0.11, 0.09, 0.06
	J	0.27, 0.25, 0.22, 0.14, 0.13, 0.11, 0.09, 0.06
	K	0.31, 0.25, 0.21, 0.14, 0.13, 0.11, 0.09, 0.07, 0.06
	L	0.31, 0.25, 0.23, 0.14, 0.13, 0.11, 0.09, 0.07, 0.06
	M	0.31, 0.28, 0.21, 0.14, 0.13, 0.11, 0.09, 0.07, 0.06
	N	0.27, 0.25, 0.21, 0.14, 0.13, 0.11, 0.09, 0.06
	O	0.31, 0.27, 0.25, 0.23, 0.11, 0.09, 0.06
	P	0.45, 0.35, 0.21, 0.16, 0.13, 0.07, 0.06, 0.05
	Q	0.45, 0.31, 0.25, 0.13, 0.09, 0.07, 0.06
	R	0.45, 0.27, 0.21, 0.13, 0.11, 0.09, 0.07, 0.06
	S	0.45, 0.37, 0.35, 0.13, 0.07, 0.06, 0.05

mtDNA was carried out as described in NAKAHARA *et al.* (2008) using PCR primers, MT1253 (NAKAHARA *et al.*, 2000), and TM-N-193 (SIMON *et al.*, 1994). The amplified fragment was treated with restriction enzymes, *Dra* I or *Ssp* I (Nippon Gene, Japan), electrophoresed on MetaPhor agarose gels (Cambrex Bio Science, Rockland, Maine), and then visualized by staining the gel with ethidium bromide according to the method described in NAKAHARA *et al.* (2008). A 100-bp or 50-bp ladder (Gibco BRL, Gaithersburg, Md.) was used as a molecular size marker.

Results

Among 513 individuals used in this study, 14 and 19 banding patterns were detected by analyses using *Dra* I and *Ssp* I, respectively. Table 2 shows length of restriction fragments included in respective banding patterns. The values were determined by comparisons of length of DNA bands observed on electrophoresed gel with those estimated from previously reported sequences of the same region (AB191470–AB191473). By combining the results obtained using these enzymes, a total of 44 haplotypes were recognized. In this study, each haplotype was denoted by two capital letters indicating banding patterns specified in Table 2.

Haplotypic composition of respective populations is shown in Table 3. In this table, among populations that had

Table 3. Haplotypic compositions of the 12 populations

Haplotypes ^a	Okinawa	Sakishima	China	Keelung	Taipei	Taichung	Taitung	Kaohsiung	Vietnam	Thailand	Davao	Philippines	Total
A-A	39	1	11	12	44	9	26	36	10	17			205
A-B					2					2			4
A-C	2				1	1		1	1	2			7
A-E						1	1						2
A-F					1			1		1			3
A-H						1				1			2
A-J					1	1			1				3
B-A	3	1	17	1	15	7	7	6	4	6			67
B-B		6	3		3		2		1	1	22	17	55
B-D		4									20	40	64
B-F					2			1		1			4
B-I					1	1	1						3
B-J					1		4		1				6
C-A					1					1			2
C-B		2			2	1	3	1					9
D-B							1			1			2
F-A					1		2						3
J-B			10		2								12
L-M					1			1					2
N-P											7	17	24
N-Q											2	5	7
Other ^b		1	3		7		1		5	7		3	27
Total	44	15	44	13	85	22	48	47	23	39	51	82	513
No. of haplotypes	3	6	5	2	22	8	10	7	9	17	4	7	44

^a Haplotype is denoted by two letters defined in Table 2.

^b Others indicate population-specific haplotypes:

Taipei (A-L, C-K, H-A, K-A, L-I, M-A, and M-J), Taitung (A-O), China (A-N X 3), Vietnam (A-I, I-J, and H-C X 3), Thailand (A-D, A-G, B-C, C-H, E-J, G-A, and H-H), Sakishima (B-E), and Philippines (B-K, N-R, and N-S).

the possibility of invading to Ryukyu Islands, populations of the Philippines (Davao and Philippines) indicated relatively characteristic haplotypic composition. It is quite simple and mostly composed of four haplotypes: B-B, B-D, N-P, and N-Q (97.7% of individuals). Populations in other Asian countries excluding the Philippines were also roughly grouped. These populations share the major haplotypes A-A and B-A, occupying 51.4% and 19.6% of individuals in this group, respectively. This group was also characterized by the existence of many minor haplotypes. Although the Okinawa population includes only three haplotypes, the composition was basically similar to this group. On the other hand, the Sakishima population was different from the Philippines and other Asian countries populations in that this population includes haplotypes specific to both of these groups; 10 of 15 individuals showed haplotypes abundantly detected from the Philippines (B-B and B-D), while several individuals showed haplotypes commonly observed in other Asian countries (A-A, B-A, and C-B). Haplotype B-B was also observed in the Taipei, Taitung, China, Vietnam, and Thailand populations. However, this haplotype was much less frequent in these populations than in the populations of the Philippines and Sakishima. Geographic distributions of all other haplotypes were separated between the Philippines and other areas excluding Sakishima.

Discussion

In this study, we found that haplotypic composition of *B. dorsalis* complex species is basically different between the Philippines and other Asian countries excluding the Sakishima region of the Ryukyu Islands. A population of the Sakishima region was composed of haplotypes specific to both of these areas. On the other hand, a population of the Okinawa region did not share any common haplotypes with the Philippines, and its composition was basically similar to

Asian countries excluding the Philippines. These results suggest that populations were basically separate between the Philippines and other Asian countries and that the Okinawa and Sakishima regions were invaded by flies from the latter and both populations, respectively. Because a small number of haplotype B-B, one of the major haplotypes in the Philippines, was detected in several other areas such as Taiwan and continental Asia including China, Vietnam, and Thailand, separation of populations between the Philippines and these areas is not so strict. It is noteworthy that the Philippines population did not share any haplotypes with these areas except in the case of B-B. Thus, the movement seems to have occurred only from the Philippines to neighboring areas. This tendency was consistent with the fact that typhoons occurring in the western Pacific region frequently move from the Philippines to Taiwan and continental areas (NYOUMURA and MIYAZAKI, 1980). Because the oriental fruit fly can fly across open sea (CHRISTENSEN and FOOTE, 1960) and *B. dorsalis* is able to survive windborne dispersal for several hundred kilometers (SHI *et al.*, 2005), individuals of haplotype B-B could also disperse for long distance between these areas.

In the analyses of nucleotide sequences of 12S rDNA (MURAJI *et al.*, 2008), individuals showing haplotypes B-B and B-D in this study were tightly grouped with individuals of *B. philippinensis*. Similarly, individuals showing N-P, N-Q, N-R, and N-S in this study were suggested to be *B. occipitalis*. Although both of these species are considered to be indigenous to the Philippines, only the former species seems to have expanded to neighboring countries. Because both of these species occur abundantly in the Philippines and are frequently detected in fruits intercepted at international airports (NAKAHARA *et al.*, 2002), this phenomenon cannot simply be explained by accidental introductions of flies due to transportation of fruit products or passengers. Thus, species-specific factors such as difference in flight activity among species could be involved in this phenomenon. Further studies are needed to ascertain the factors affecting invasions of fruit flies of this group.

In this study, major haplotypes such as A-A and B-A, commonly distributed in Taiwan and continental Asia, were also detected from Okinawa and Sakishima populations of the Ryukyu Islands. This phenomenon indicates that these populations included migrants from Taiwan and/or continental Asia. However, we could not determine their geographic origins precisely. This was due to the lack or scarcity of geographic population structures among possible flight origins excluding the Philippines. A similar tendency was also reported for populations of this species in southwestern China (SHI *et al.*, 2005). Thus, populations of the *B. dorsalis* complex in these areas might be intermingled by factors such as insect movement. This phenomenon must have been attained by the significant expansion of this species in the last century. To solve relationships between flies in the Ryukyu Islands and these areas, it may be useful to apply highly polymorphic genetic markers like microsatellite DNA (DAI *et al.*, 2004; AKETARAWONG *et al.*, 2007).

In this study, a close relationship between populations of the Philippines and the Sakishima region of the Ryukyu Islands was detected. This result was consistent with our previous study (MURAJI *et al.*, 2008) based on the analyses of PCR-RFLP and nucleotide sequences of the mitochondrial 12S rDNA. In that report, we concluded that most individuals collected in the Sakishima region must be migrants that flew directly from the Philippines because the common haplotype between these areas was not detected from 257 individuals collected in Taiwan and neighboring countries. This hypothesis was also supported by circumstantial evidence including meteorological factors in this area and the position of the Sakishima region in international transportation. On the other hand, the present study showed that one of the two haplotypes common between the Sakishima region and the Philippines, B-B, was also distributed in Taiwan and continental Asia. However, because this haplotype is very rare in these areas, it seems unlikely that fruit flies of these areas had invaded the Sakishima region. This idea is supported by the fact that another common haplotype, B-D, was not detected from these regions.

In order to confirm the factor affecting the close relationship between the Philippines and the Sakishima region, it is important to accumulate data on the relationship between meteorological factors and the number of trapped insects showing *B. philippinensis*-specific haplotypes in the Sakishima region. These data can be used to confirm the hypothesis on migrations between these areas and to develop a method to predict their invasion into the Sakishima region.

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和 文 摘 要

琉球諸島及びその近隣諸国から収集されたミカンコミバエ種群の
ミトコンドリアDNA A-Tリッチコントロール領域における
PCR-RFLP解析中原重仁・村路雅彦¹⁾横浜植物防疫所調査研究部, ¹⁾独立行政法人 農業生物資源研究所

琉球諸島で時折誘殺されるミカンコミバエ種群の侵入源を明らかにするため、琉球諸島及びその近隣諸国の合計16地点から収集した513個体について、ミトコンドリアDNA A-Tリッチコントロール領域のPCR-RFLP解析を行った。制限酵素 *Dra* Iと *Ssp* Iにより、合計44種類のハプロタイプが確認されたが、沖縄諸島と先島諸島で検出されたハプロタイプはそれぞれ3種類と6種類のみであった。沖縄諸島で捕獲された個体はフィリピンを除くアジア地域に共通のハプロタイプで構成されていたが、先島諸島で捕獲された個体はフィリピンとの高い関係性が示され

た。これらの結果から、琉球諸島への飛来ルートは少なくとも2つあると考えられた。このことは、*Bactrocera philippinensis*が先島諸島へ飛来しているとするこれまでの研究結果を支持するものであった。また、フィリピンの *B. philippinensis*で検出された2つのハプロタイプのうちの1つは、少ないながらも台湾、中国、ベトナム、タイの広範な地域にわたって確認された。この現象は、フィリピンに分布する本種が、これらの地域に分布を拡大している可能性を示唆すると考えられた。